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Prion Protein Devoid of the Octapeptide Repeat Region Restores Susceptibility to Scrapie in PrP Knockout Mice

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Summary

Mice devoid of PrP are resistant to scrapie and fail to replicate the agent. Introduction of transgenes expressing PrP into such mice restores susceptibility to scrapie. We find that truncated PrP devoid of the five copper binding octarepeats still sustains scrapie infection; however, incubation times are longer and prion titers and protease-resistant PrP are about 30-fold lower than in wild-type mice. Surprisingly, brains of terminally ill animals show no histopathology typical for scrapie. However, in the spinal cord, infectivity, gliosis, and motor neuron loss are as in scrapie-infected wild-type controls. Thus, while the region comprising the octarepeats is not essential for mediating pathogenesis and prion replication, it modulates the extent of these events and of disease presentation.

Introduction

PrP, the prion protein, plays a central role in the pathogenesis of transmissible spongiform encephalopathies such as scrapie or bovine spongiform encephalopathy (BSE) (Prusiner, 1996, 1998; Weissmann, 1999; Weissmann et al., 1996). The normal form of PrP, designated PrP^{C}, is encoded by a single-copy gene (Basler et al., 1986) and is expressed in the brain of healthy and prion-infected organisms to about the same extent (Chesebro et al., 1985; Oesch et al., 1985). There is overwhelming evidence that a modified form of PrP^{C}, which we designate as PrP* (Weissmann, 1991), is the principal if not the only component of the infectious agent, or prion and that it is devoid of nucleic acid. The "protein-only" hypothesis (Griffith, 1967) states that the abnormal form of PrP propagates by interacting with PrP^{C} and converting it into a likeness of itself (Prusiner, 1989, 1996; Weissmann et al., 1996). It has been proposed that a partially protease-resistant, aggregated form of PrP, named PrP^{Sc} or PrP-res, is congruent with PrP*, mainly because purification of infectivity leads to enrichment of PrP^{Sc} and because both infectivity and PrP^{Sc} are largely resistant to proteinase K digestion (Prusiner, 1989). However, in some instances, brains of animals or humans suffering from prion disease are devoid of detectable levels of protease-resistant PrP (Collinge et al., 1995; Telling et al., 1996; Lasmezas et al., 1997; Manuelidis et al., 1997; Manson et al., 1999); the significance of this finding must be tempered by the consideration that the detection limit for infectivity is three to five orders of magnitude lower than for PrP^{Sc}.

One of the more remarkable features of human prion diseases is that they arise not only as a consequence of transmission, but also of mutations in the PrP gene (Hsiao et al., 1989; Collinge and Palmer, 1994; Parchi and Gambetti, 1995; Collinge, 1997; Prusiner and Scott, 1997). In addition to about 20 different point mutations associated with Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) in man, there are also amplifications of a stretch of octarepeats in the amino-proximal region of PrP from normally 5 to as many as 14 (Krasemann et al., 1995; Collinge, 1997), suggesting that this region of PrP might be important for the spontaneous conversion event.

Mice devoid of PrP develop and behave normally (Büeler et al., 1992) but are resistant to prion disease (Büeler et al., 1993; Manson et al., 1994; Sailer et al., 1994; Sakaguchi et al., 1995). Moreover, introduction of PrP transgenes into such *Prnp*$^{o/o}$ mice restores susceptibility to scrapie (Fischer et al., 1996), thus paving the way to structure–function analyses of PrP. In earlier experiments, we showed that in *Prnp*$^{o/o}$ mice overexpression of truncated PrP transgenes encoding PrP with a deletion of codons 32–80 (inclusive) and therefore retaining only one of the five octarepeats, sustained replication of infectious agent and development of disease (Fischer et al., 1996).

We now show that PrP with a deletion of codons 32–93 (inclusive), and thus devoid of all five octarepeats, also restores susceptibility to scrapie in PrP knockout mice. However, incubation times are longer and result in prion titers in brain and spleen that are lower than in wild-type mice in all stages of the disease. Interestingly, even in terminally ill mice, no histopathological changes were evident in the brain at the level of light microscopy; however, there was neuronal loss and astrogliosis in the cervical spinal cord. Thus, while the octarepeat sequence is not essential for sustaining prion replication and disease, it does affect the level of prion accumulation and pathogenesis in the brain.

Results

Generation and Characterization of PrP Knockout Mice Transgenic for PrP with a Deletion of Amino Acids 32–93

We prepared two mouse lines, *C4/C4* and *C15/C15*, expressing truncated PrP devoid of the five octarepeats

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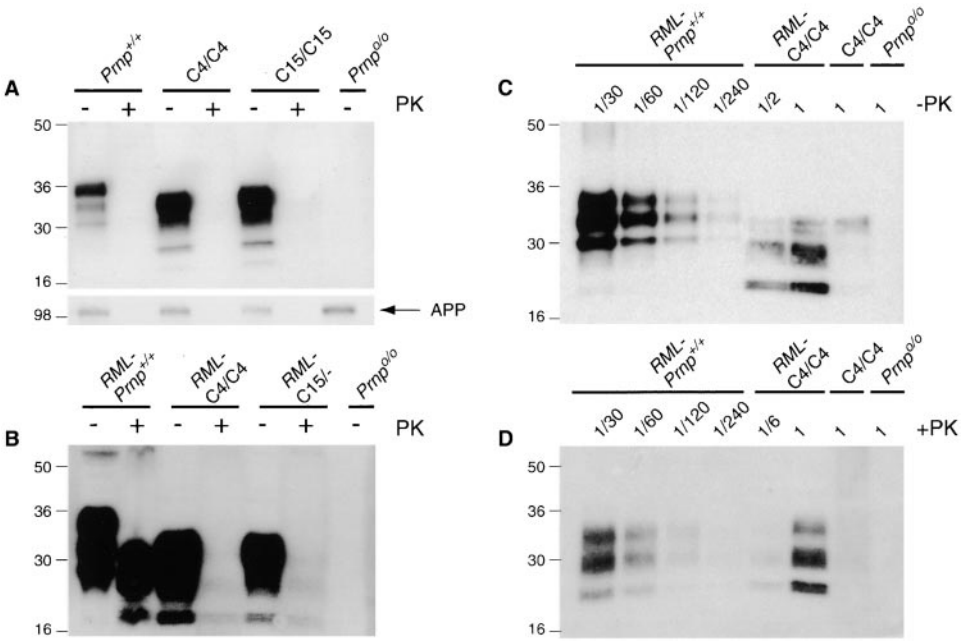

Figure 1. Western Blot Analysis of Total, Phosphotungstate-Precipitable, and Protease-Resistant PrP

Samples from uninfected or RML-infected, terminally ill mice were analyzed by Western blotting before (A and B) or after phosphotungstate (NaPTA) precipitation (C and D) as described in Experimental Procedures, using antisera 6H4 for PrP.

(A) Brain homogenates from uninfected mice; the blots were also stained with antibody 22C11 for APP, which served as internal control. Quantification was as described in the Experimenal Procedures section.

(B) Brain homogenates from RML-infected, terminally ill mice without (−) and after (+) proteinase K treatment.

(C) Brain homogenates of noninoculated *PrP*$^{o/o}$ and *C4/C4* mice or RML-infected, terminally ill wild-type and *C4/C4* mice were subjected to the NaPTA precipitation procedure. The pellets were resuspended in sample buffer with 8 M urea without prior protease treatment (−PK).

(D) Samples were prepared and analyzed as in (C), but pellets were resuspended in 0.1% Sarkosyl and treated with proteinase K (+PK) before loading.

In both (C) and (D), aliquots of 22 μl (lanes marked 1) or the fraction thereof as indicated were subjected to Western blot analysis. Except for the *C15* mouse in (B), all mice were homozygous for the transgene.

(PrPΔ32–93) on a *Prnp*$^{o/o}$ background, using the "half genomic" PrP vector in which the reading frame is under control of the PrP promoter (Shmerling et al., 1998). Both lines of mice, homozygous for the transgene cluster, expressed the mutant protein in brain at about 4-fold higher level than wild type (Figure 1A; Table 1). The distribution of the truncated PrP in brain and brainstem was similar to that of full-length PrP in wild-type mice (Figure 2A). Mice of both lines remained healthy without any neurological signs for over 800 days.

Susceptibility to Mouse Prions of PrP Knockout Mice Transgenic for PrPΔ32–93

About 200–300 days after intracerebral (i.c.) inoculation with mouse-adapted prions (Table 1), hemizygous (*C4/−; C15/−*) as well as homozygous (*C4/C4; C15/C15*) mice presented with scrapie-like symptoms similar to those of wild-type mice, including ataxia and kyphosis, except that both lines developed front leg paresis in the late stages of the disease, while the hind legs seemed less affected.

Brain sections of six terminally ill animals (two of each *C4/C4*, *C15/C15*, and *C4/−*) were examined for pathological changes. Neither spongiosis nor gliosis was evident in the thalamus (Figures 3D and 3E), brainstem (Figures 3K and 3L), or hippocampus (data not shown) of transgenic mice, while strong spongiosis and gliosis were found in terminally ill wild-type mice and transgenic mice overexpressing full-length PrP (*tga20/tga20)* or PrP retaining only one octarepeat (*tgd12/tgd12* [Fischer et al., 1996]) (Figures 3A–3C and 3H–3J). Moreover, no alterations were found in skeletal muscle and peripheral nerves (data not shown). However, strong astrogliosis and spongiosis were seen in the spinal cord of terminally ill mice expressing PrPΔ32–93, as in wild-type PrP (Figure 4A). Moreover, there was a reduction in the number of motor neurons in the Rexed laminae VIII and IX of the spinal cord. In the cervical spinal cord levels C4–C8, the motor neuron reduction of about 10%–25% in infected *C4/C4* mice was similar to that of infected wild-type and *tga20/tga20* mice, as compared with age-matched mock infected mice (Figure 4B). No significant loss of motor neurons was found in the thoracic (T1) and lumbar (L1–L4) spinal cord levels of infected mice of the three genotypes (data not shown).

Western blot analysis of brain tissue of terminally ill *C4/C4* and *C15/−* mice revealed only traces of protease-resistant PrP (Figure 1B). This finding could come about either because less PrP^{Sc} accumulated or because PrP^{Sc}Δ32–93 was less resistant to proteinase K digestion. In order to compare the level of PrP^{Sc}Δ32–93 with that of full-length PrP^{Sc} without making use of protease digestion, we made use of the finding that full-length PrP^{Sc} and its protease-resistant moiety, PrP27–30, which lacks 60 amino-terminal residues, are precipitated by sodium phosphotungstate (NaPTA) with the same efficiency, while PrP^{C} is essentially not precipitated (Safar et al., 1998). We added 1.8 mg total protein from brains

Table 1. Response of Mice with Various PrP Genotypes to Intracerebral Inoculation with Mouse Scrapie Prions

Inoculum	Recipient Mouse Line [Genotype]	Gene copies[a]	PrP Level[b]	Days to Symptoms ± SD (n)	Days to Terminal Disease ± SD (n)
RML	C4/− [*Prnp*$^{o/o}$, *tg*(*PrPΔ32–93*/−)]	25	4[c]	232 ± 14 (4)	257 ± 7 (4)
RML	C4/C4 [*Prnp*$^{o/o}$, *tg*(*PrPΔ32–93/PrPΔ32–93*)]	n.d. (50)	4[d]	219 ± 20 (6)	232 ± 21 (6)
RML C4[h]	C4/C4 [*Prnp*$^{o/o}$, *tg*(*PrPΔ32–93/PrPΔ32–93*)]	n.d. (50)	4[d]	232 ± 20 (6)	244 ± 16 (6)
RML	C15/− [*Prnp*$^{o/o}$, *tg*(*PrPΔ32–93*/−)]	25	3[c]	313 ± 23 (5)	371 ± 15 (3)
RML	C15/C15 [*Prnp*$^{o/o}$, *tg(PrPΔ32–93/PrPΔ32–93)*]	n.d. (50)	3.8[d]	214 ± 41 (3)	239 ± 45 (3)
RML	wild type; C57BL/6 × 129/Sv [*Prnp*$^{+/+}$]	2[e]	1[e]	158 ± 11 (31)[f]	171 ± 11 (31)[f]
RML	PrP hemizygous [*Prnp*$^{+/o}$]	1[e]	0.5[e]	290 ± 33 (19)[g]	415 ± 30 (15)[g]

[a] Relative to wild type; determined by quantitative PCR. In parentheses, extrapolated from hemizygous animals.
[b] Relative to wild type; determined by densitometric analysis of Western blots.
[c] PrP was detected with a polyclonal antibody R340 (Brandner et al., 1996), and signals were quantified relative to those of wild-type mice (without internal control).
[d] PrP was detected with a monoclonal antibody 6H4 (Prionics AG, Switzerland), and signals were quantified in comparison to those of wild-type mice, with APP as internal control (see Figure 1A).
[e] By definition.
[f] (Büeler et al., 1993).
[g] (Büeler et al., 1994).
[h] RML C4 prions are from RML-infected terminally ill C4/C4 mice.
n, Number of animals; n.d., not done.

of terminally ill, RML-infected wild-type or *C4/C4* mice to a 10% homogenate of *Prnp*$^{o/o}$ brain, carried out the phosphotungstate precipitation, and compared the amounts of PrP in the pellets both before and after digestion with proteinase K. Before digestion, there was about 50 times less NaPTA-precipitable PrPΔ32–93 than

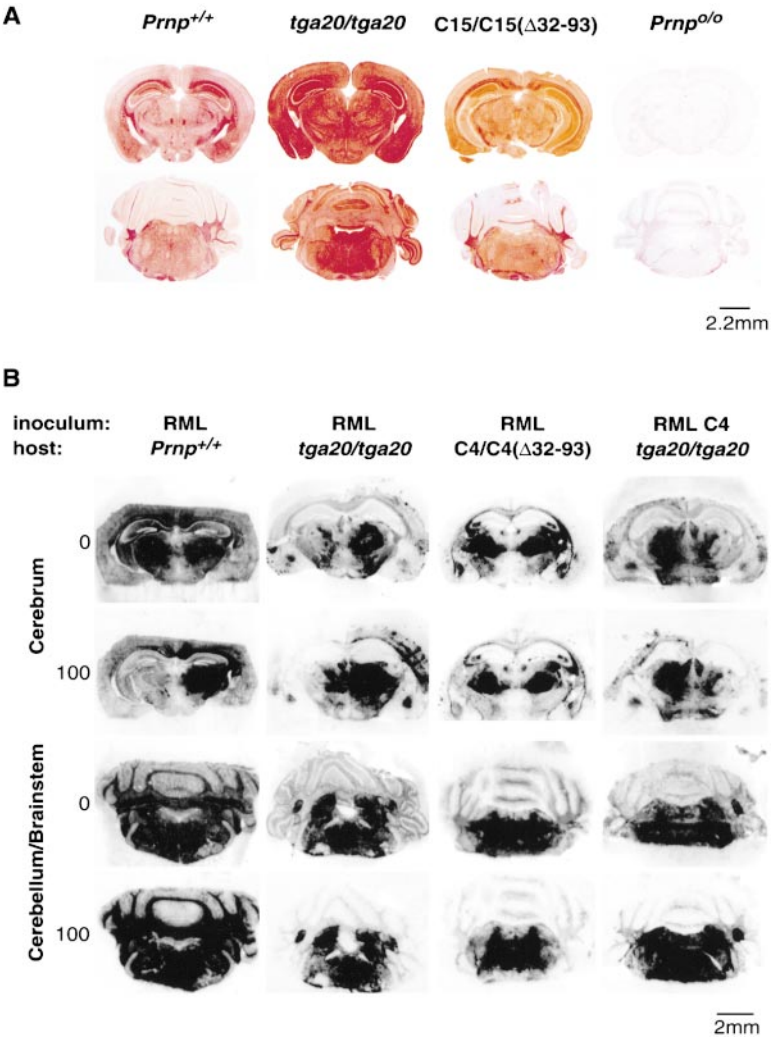

Figure 2. Distribution of Total PrP and PrP^{Sc} in Coronal Brain Sections of Wild-Type and Transgenic Mice

(A) Immunostaining of PrP cerebrum (upper row) and brainstem–cerebellum of uninfected mice (lower row). Truncated PrP (third column) is expressed in a similar pattern as full-length PrP in wild-type brain.

(B) Histoblots of cerebrum (upper two rows) and cerebellum with brainstem (lower two rows) of terminally ill, RML-infected mice. Blots were treated with 100 μg/ml proteinase K (100) or not treated (0). While PrP^{Sc} is present throughout the cerebrum, cerebellum, and brainstem of RML-infected, wild-type mice (first column), it is found mainly in the thalamus and brainstem of terminally ill *tga20/tga20* mice (second column) and *C4/C4* mice (third column) inoculated with RML scrapie. Brains of *tga20/tga20* mice (fourth column) infected with 1% brain homogenate from terminally ill *C4/C4* mice (which had originally been infected with RML inoculum) exhibit proteinase K–resistant PrP immunostaining indistinguishable from that of RML-infected *tga20/tga20* brains (second column).

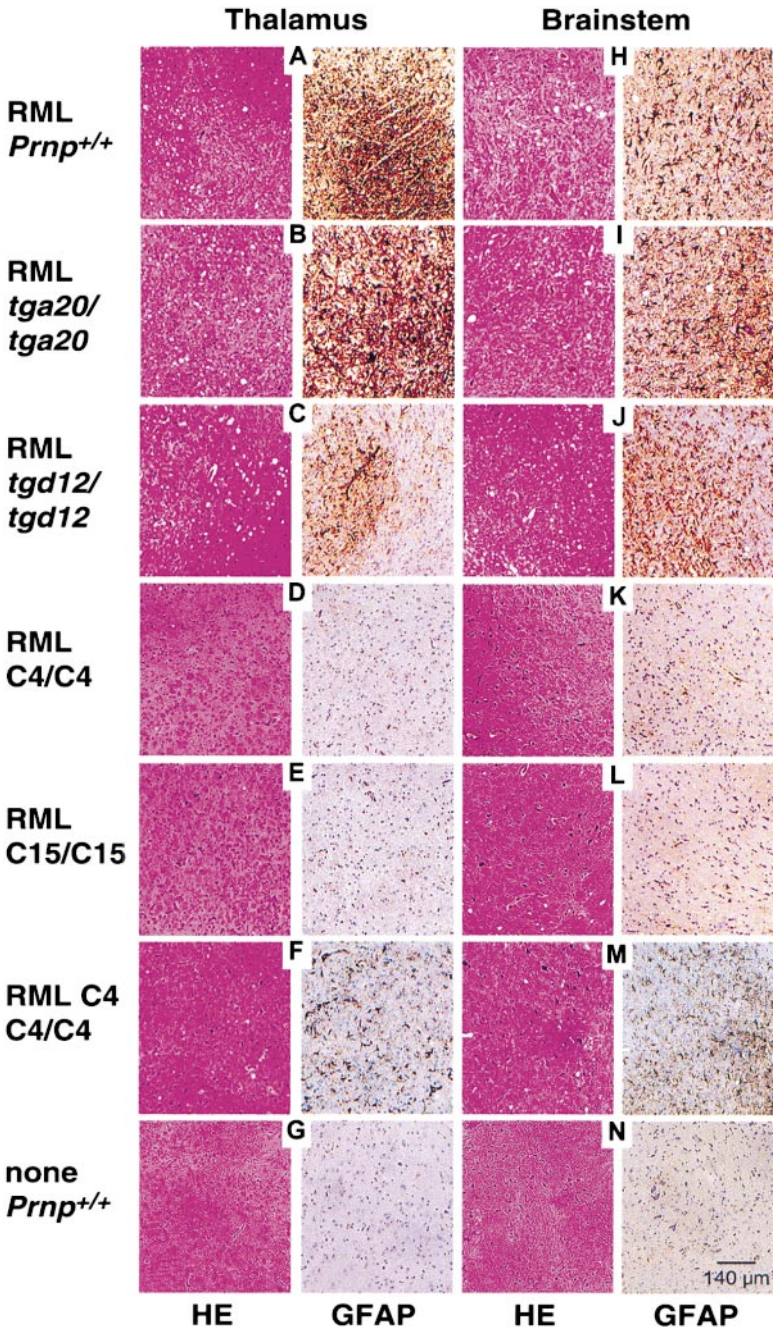

Figure 3. Brain Sections of Terminally Ill Mice Expressing Wild-Type or Amino-Proximally Truncated PrP

Coronal sections of thalamus (A–G) and brainstem (H–N) were stained with hematoxylin–eosin (HE) or immunostained for glial fibrillary acidic protein (GFAP). At the left of each row, the inoculum and the line of the experimental animal are indicated. RML-infected, wild-type mice (A and H), *tga20/tga20* mice (overexpressing wild-type PrP; [B and I]), and *tgd12/tgd12* (overexpressing PrPΔ32–80 [Fischer et al., 1996]; [C and J]) show spongiosis (HE) and pronounced astrocytosis (GFAP) in both brainstem and thalamus. RML-infected *C4/C4* (D and K) or *C15/C15* (E and L) mice that overexpress PrPΔ32–93 and are devoid of all octarepeats show no evident histopathology. Some perivascular shrinkage in the brainstem of *C15/C15* mice (L) can be distinguished from spongiosis at higher magnification (data not shown). *C4/C4* mice inoculated with prions passaged once through *C4/C4* mice exhibited no spongiosis and only moderate gliosis in the thalamus (F), but mild spongiosis and reactive astrocytic gliosis were evident in the brainstem (M). Uninfected wild-type mice (G and N) served as negative controls.

full-length PrP (Figure 1C), and after digestion, about 30 times less (Figure 1D). This means that the proportion of digestible PrP is about the same for the wild type and the *C4/C4* sample (Figures 1C and 1D cannot be compared directly because the blots resulted from different experiments). Thus, we conclude that the low level of protease-resistant PrPΔ32–93 reflects a low rate of accumulation and not an increased susceptibility to proteolysis.

The distribution of protease-resistant PrPΔ32–93 as judged by (nonquantitative) histoblot analysis was similar to that in terminally ill transgenic mice overexpressing full-length PrP (*tga20/tga20*) (Figure 2B). However, the intensity of staining of wild-type and mutant samples did not reflect the 30-fold lower levels of the truncated PrP found by Western analysis of homogenate supernatants. Examination of the low-speed pellets resulting from the centrifugation of the initial brain homogenates (which are usually discarded) excluded the possibility that truncated PrP^{Sc} was segregated into that fraction, confirming the previous conclusions. Most likely, the signal intensities in the histoblot are not linear with the amount of PrP^{Sc} present.

Prion Titers in Scrapie-Infected PrP Knockout Mice Expressing PrPΔ32–93

Prion titers in spleen and brain of infected animals were determined by inoculating homogenates i.c. into *tga20/*

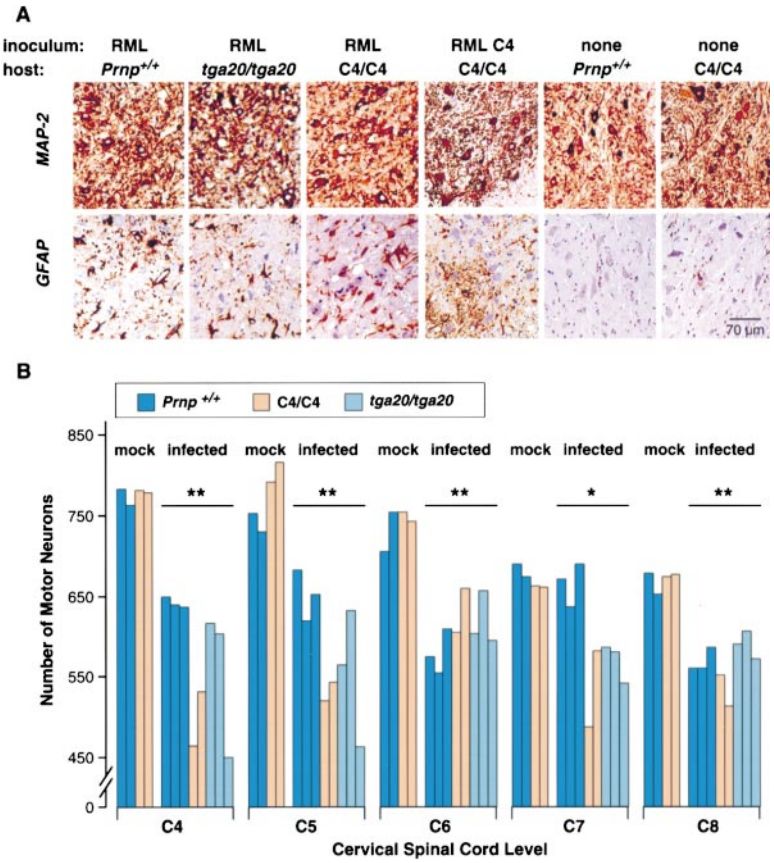

Figure 4. Sections of the Cervical Spinal Cord Wild-Type and Transgenic Mice

(A) Coronal sections showing the Rexed layer IX of the cervical spinal cord level C4 of terminally ill scrapie-infected *Prnp*$^{+/+}$, *tga20/tga20*, and *C4/C4* mice. Motor neurons were identified by their large size, pale nuclei, and content of microtubule-associated protein 2 (MAP-2), as determined by immunohistochemistry. The pathological changes in *C4/C4* mice inoculated with either RML or RML C4 prions (prions derived from RML-inoculated *C4/C4* mice) were indistinguishable from RML-infected wild-type and *tga20/tga20* mice as judged by immunostaining for GFAP. There is no gliosis or loss of motor neurons in uninfected *C4/C4* mice compared to wild-type controls.

(B) Graphic representation of the number of motor neurons in the Rexed layers VIII and IX in the ventral spinal cord from levels C4–C8 of RML-infected *Prnp*$^{+/+}$, *C4/C4*, and *tga20/tga20* mice. The statistical significance of motor neuron loss in infected and uninfected animals was analyzed separately for each cervical spinal cord segment by parametric statistics (two-tailed t test). The number of motor neurons in the set of infected mice of all genotypes is reduced by about 10%–25% compared with the set of uninfected mice of the same age. Differences between infected mice of the various genotypes are not statistically significant. **$p < 0.0005$, *$p < 0.025$.

tga20 indicator mice (Table 2). A mouse homozygous for the *Prnp*$^{\Delta 32-93}$ cluster (*C4/C4*), sacrificed 4 months after inoculation, had a titer of about 2 log LD_{50} U/ml 10% brain homogenate but no significant infectivity in the spleen (Table 2). After 8 months, when clinical symptoms were apparent, the titer in the pooled brain homogenates of 2 *C4/C4* mice was about 7 log LD_{50} U/ml 10% homogenate and in the spleen infectivity was at borderline detectability (about 2.5 log LD_{50} U/ml 10% homogenate). Similarly low titers were also found in brain and spleen of a terminally ill *C15/−* mouse analyzed (Table 2). No significant infectivity could be detected in the spleen of *C15/C15* mice 4 weeks after i.c. inoculation, a time at which prion levels in the spleen reached close to maximal value in infected wild-type mice (Büeler et al., 1993). Thus, 4 weeks after i.c. inoculation, spleens of such transgenic mice contained at least 10^5 times less prions than those of wild-type mice (5.2 log LD_{50} U/ml 10% homogenate), showing that truncation also dramatically affects prion accumulation in the spleen.

Transmission of 30 µl 1% homogenate of cervical and thoracic spinal cord segments of two terminally ill *C4/C4* mice into the brains of indicator mice caused terminal disease after 63 ± 2 days (4/4) and 69 ± 1 days (4/4), respectively. Similar incubation times were found after transmission of cervical and thoracic spinal cord segments of two terminally ill wild-type mice, namely 69 ± 1 (3/3) and 69 ± 0 days (4/4), respectively. Thus, although infectivity titers are about 30 times lower in brains of RML-inoculated *C4/C4* compared to wild-type mice, they are the same in spinal cord.

PrPΔ32–93-Expressing Brain Grafts Propagate Prions and Remain Devoid of Scrapie-like Pathology

To further investigate pathogenesis in brain tissue expressing PrPΔ32–93, embryonal neuroectodermal tissue from *C4/C4* mice was grafted (Isenmann et al., 1996) into the caudoputamen of adult *Prnp*$^{o/o}$ mice and inoculated i.c. with RML prions (Figure 5). As reported earlier, *tga20/tga20* grafts (6/6) showed strong neuronal loss, spongiosis, and gliosis by 200 days after inoculation with mouse prions (Brandner et al., 1996), while *C4/C4* grafts (10/10), 268 days after inoculation, showed no pathological changes, although they differentiated into neuronal and glial tissue, as evidenced by synaptophysin and GFAP staining, and accumulated PrP^{Sc} (Figure 5). These findings confirm the results described above, namely that no pathological changes could be observed by light microscopy in brain tissue from scrapie-infected mice expressing PrP devoid of the octarepeat region. Four such infected *C4/C4* grafts were recovered, homogenized, and each homogenate inoculated into four *tga20/tga20* indicator mice. In all cases (16/16), terminal scrapie resulted after 102 ± 16 days. Two RML-infected *tga20/tga20* graft homogenates caused terminal disease in indicator mice (8/8) after 66 ± 4 days, reflecting the higher titers attained in brains of *tga20/tga20* mice as compared to those of *C4/C4* mice. Because no infectivity was found in two PrP-deficient grafts 226 days

Table 2. Infectivity in Organs of Prion-Inoculated Mice[a]

Inoculum	Inoculated Mouse Line [Genotype]	Months p.i.	Dilution	Scrapie-Ill Recipients n/n_o (days p.i. ± SD) Spleen	Brain	Titer[b] Spleen	Brain
Preclinical Stage							
RML	C4/− [*Prnp$^{o/o}$, tg(PrPΔ32–93/−)*]	2	10^{-1}	0/4 (>200)	4/4 (112 ± 14)	<1	2.2
RML	C4/C4 [*Prnp$^{o/o}$, tg(PrPΔ32–93/PrPΔ32–93)*]	4	10^{-1}	1/8 (113)	10/10 (115 ± 16)	<1.5	1.9
RML	C15/C15 [*Prnp$^{o/o}$, tg(PrPΔ32–93/PrPΔ32–93)*]	1	10^{-1}	0/4 (>200)	n.d.	<1	n.d.
RML	wild type [*Prnp$^{+/+}$*] (2 spleens pooled)	1	10^{-1}	3/3 (75 ± 1)	n.d.	5.2[c]	n.d.
			10^{-3}	4/4 (91 ± 10)			
			10^{-4}	1/4 (94)			
			10^{-5}	0/5 (>200)			
Terminal stage							
RML	C4/C4 [*Prnp$^{o/o}$, tg(PrPΔ32–93/PrPΔ32–93)*] (2 brains, 2 spleens pooled)	8	10^{-1}	2/4 (111 ± 6)	4/4 (68 ± 2)	<2.5	7.0[c]
			10^{-3}		4/4 (80 ± 5)		
			10^{-4}		4/4 (90 ± 6)		
			10^{-5}		4/4 (114 ± 12)		
			10^{-6}		0/4 (>130)		
			10^{-7}		0/4 (>130)		
RML C4[d]	C4/C4 [*Prnp$^{o/o}$, tg(PrPΔ32–93/PrPΔ32–93)*] (2 brains pooled)	8	10^{-1}	n.d.	4/4 (68 ± 1)	n.d.	6.7[c]
			10^{-3}		4/4 (88 ± 6)		
			10^{-4}		4/4 (100 ± 9)		
			10^{-5}		2/4 (102 ± 11)		
			10^{-6}		1/5 (97)		
			10^{-7}		0/4 (>130)		
RML	C15/−[*Prnp$^{o/o}$, tg(PrPΔ32–93/−)*]	10	10^{-1}	2/4 (113 ± 6)	3/3 (69 ± 2)	<2.5	7.3
			10^{-2}	0/2 (>200)	4/4 (73 ± 3)		
			10^{-3}		4/4 (84 ± 5)		
RML	wild type [*Prnp$^{+/+}$*] (2 brains pooled)	5	10^{-1}	n.d.	3/3 (65 ± 10)	n.d.	8.2[c]
			10^{-3}		4/4 (65 ± 5)		
			10^{-5}		4/4 (88 ± 3)		
			10^{-6}		3/3 (92 ± 15)		
			10^{-7}		1/4 (129)		
			10^{-8}		0/4 (>200)		

[a] Unless stated otherwise, one organ was assayed. Of the indicated dilution of a 10% tissue homogenate 30 μl was injected i.c. into *tga20/tga20* mice. Animals were observed for 200 days. n/n_o, number of animals acquiring scrapie/number of animals inoculated. n.d., not done.
[b] Titers (log LD_{50} U/ml of 10% homogenate) were determined by the incubation time method (Prusiner et al., 1982) using the standard curve for *tga20/tga20* (Brandner et al., 1996). Limit of detection: ~1 log LD_{50} U/ml 10% homogenate.
[c] Titer calculated by endpoint titration (Reed and Muench, 1938).
[d] RML C4 prions are from RML-infected terminally ill C4/C4 mice.

after RML inoculation, assayed in four mice each (0/8), it is unlikely that infectivity was due to residual inoculum.

Properties of Prions from RML-Infected Transgenic Mice Expressing PrPΔ32–93 Passaged through Mice Expressing the Same Transgene

Prions from a donor whose PrP sequence differs from that of the recipient may cause disease after a much longer incubation time than prions from a donor with the same sequence, a phenomenon designated as "prion transmission barrier" and considered to be equivalent to the naturally occurring "species barriers" (Scott et al., 1989). Because the difference between PrPΔ32–93 and wild-type PrP might cause a prion transmission barrier, brain homogenates pooled from two RML-infected, sick *C4/C4* mice were inoculated into *C4/C4* mice to overcome such a conjectural barrier. However, all inoculated mice (6/6) succumbed to scrapie after 244 ± 16 days, that is, after a delay similar to that resulting after RML infection, namely 232 ± 21 (Table 1). Thus, RML passaged once through *C4/C4* mice did not result in reduced incubation time; however, it did give rise to moderate spongiosis in the brainstem (Figure 3M) and mild reactive astrocytosis in thalamus and brainstem of three *C4/C4* mice analyzed (Figures 3F and 3M), in contrast to RML-infected *C4/C4* or *C15/C15* mice, where no pathology was detected (Figures 3D and 3K or 3E and 3L, respectively). The distribution of PrP^{Sc} in brains of *C4/C4* mice, as judged by histoblotting, was similar whether inoculation was with RML prions or with prions derived from RML-infected *C4/C4* mice (data not shown). Inoculation of mice expressing wild-type PrP (*tga20/tga20*) with 1% brain homogenates from RML- or RML-C4-infected, sick mice caused scrapie after similar incubation times, namely 68 days (Table 2).

Estimated titers in brains of *C4/C4* mice inoculated with RML or *C4/C4*-passaged RML were about 7 and 6.7 LD_{50} U/ml 10% homogenate, respectively. Thus, the moderate pathological changes in the brains of *C4/C4* mice inoculated with *C4/C4*-passaged RML were not associated with a higher prion titer than that in RML-inoculated *C4/C4* mice, which had no discernible brain pathology (Table 2). In this regard, passaging RML once through *C4/C4* mice did not change its properties.

Discussion

The N-terminal half of PrP contains a conserved region of tandem repeats of an eight amino acid sequence

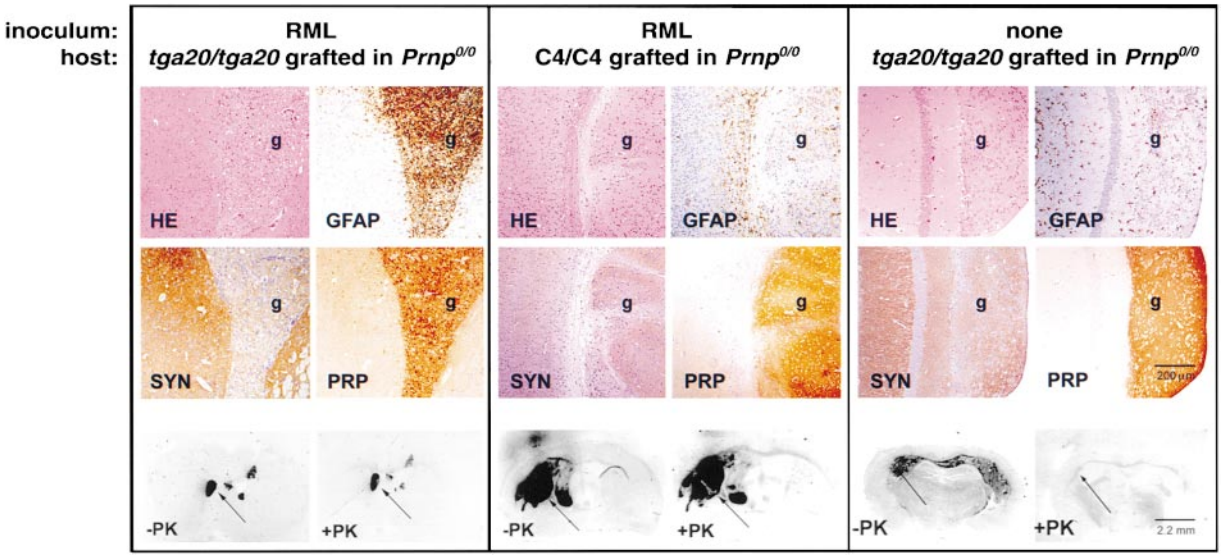

Figure 5. Immunohistochemistry and Histoblots of RML-Infected and Uninfected Neuroectodermal Grafts Derived from *tga20/tga20* and *C4/C4* Embryos in *Prnp*$^{o/o}$ Mouse Brain

Engrafted mice were prepared and inoculated as described in the Experimental Procedures. Deparaffinized brain sections were stained with hematoxylin–eosin (HE) or antibodies to GFAP. There was significant spongiosis and astrogliosis in *tga20/tga20* grafts (200 days after inoculation; left) but not in *C4/C4* grafts (268 days after inoculation; middle) or in uninfected *tga20/tga20* grafts (286 days after transplantation, right). Synaptophysin immunoreactivity (SYN) in *C4/C4* grafts documents full differentiation into neuronal tissue and remained unchanged in infected *C4/C4* grafts. Synaptophysin was reduced in infected *tga20/tga20* grafts, indicating neuronal damage. PrP (PRP) immunoreactivity reveals prominent punctate deposits in RML-infected *tga20/tga20* grafts but only few deposits in RML-infected *C4/C4* and none in uninfected *tga20/tga20* grafts. PrP was detected by polyclonal antisera R340 and visualized using the Tyramide Signal Amplification kit (NEN, Life Science, Boston). The bottom row shows histoblots of the three groups with (+PK) or without (−PK) proteinase K digestion. Proteinase K–treated histoblots display intense PrP immunostaining in both infected grafts but not in uninfected *tga*20/*tga*20 grafts (arrows).

(octarepeats) with affinity for copper ions (Hornshaw et al., 1995; Viles et al., 1999). Amplification of the repeat number beyond the usual five has been found in association with human familial prion diseases (Collinge, 1997), and the number of repeats appears to determine the type of cerebellar amyloid deposits (Vital et al., 1998).

In this paper, we asked the question of whether the octarepeat region of PrP is essential for sustaining prion replication and scrapie disease. We therefore introduced into *Prnp*$^{o/o}$ mice PrP transgenes from which the segment comprising codons 32–93 had been deleted. The resulting mature protein contained the first nine amino acids (retained, albeit unnecessarily as later emerged [Supattapone et al., 1999], to ensure correct posttranslational processing) and was devoid of all octarepeats. Two lines of mice homozyous for the transgene cluster, *C4/C4* and *C15/C15*, expressed the truncated PrP at about four times wild-type level and with the same overall distribution pattern as in wild type. Mice hemizygous or homozygous for the cluster were challenged with mouse prions. All animals succumbed to scrapie-like disease, showing that the truncated PrP was competent in this regard. Interestingly, the animals showed front leg paresis rather than the usual hind leg symptoms. Incubation times to first symptoms (31–45 weeks) and to terminal disease (34–53 weeks) were longer than for wild-type controls (22.5 and 24.5 weeks, respectively), albeit shorter than for hemizygous *Prnp*$^{o/+}$ mice (41 and 60 weeks, respectively) (Büeler et al., 1994; Manson et al., 1994). While, in wild-type mice, accumulation of prions and PrP^{Sc} is followed within weeks by clinical symptoms and death, hemizygous PrP knockout mice accumulate high levels of infectivity and PrP^{Sc} by 20–24 weeks and yet remain free of symptoms for 30 weeks thereafter (Büeler et al., 1994). The prion titers in the brains of mice expressing PrP devoid of all five octarepeats (PrPΔ32–93) were lower at all stages of the disease; in terminally sick mice, infectivity was about 10–30 times and protease-resistant and NaPTA-precipitable PrP about 30–50 times lower than in their wild-type counterparts. Because the transgenic mice expressed about four times more PrP than wild-type mice and showed the same expression pattern, we conclude that the long incubation times and low prion and PrP^{Sc} levels are the consequence of the deletion. Considering that the scrapie form of truncated PrP has the same degree of resistance to protease as that of full-length PrP, it seems unlikely that the low prion and PrP^{Sc} levels are the result of more rapid turnover; rather, we believe that they are the consequence of a decrease in the conversion rate of the truncated PrP.

Strikingly, we were unable to discern histopathological lesions in brain and brainstem of terminally ill PrPΔ32–93 animals at the level of light microscopy, not even the astrogliosis that so far has been found in all murine scrapie disease. On the other hand, infectivity, gliosis, and loss of motor neurons in the cervical segment of the spinal cord were similar in *C4/C4* and wild-type mice or in transgenic mice overexpressing full-length PrP (*tga20/tga20*).

It is remarkable that lethal disease developed at such low overall levels of PrP^{Sc} in the brain, raising once more the question as to whether the protease-resistant form of PrP plays a direct pathogenetic role in brain. *Tga20/*

tga20 mice overexpressing full-length PrP 10-fold have a vastly reduced incubation time (about 65 days) as compared to wild-type mice with a similar genetic background (about 170 days), and at preterminal disease have at least equal prion titers as their wild-type counterparts but lower levels of PrP^{Sc} (Fischer et al., 1996). Previous reports have shown that extracellular PrP^{Sc} does not give rise to pathological changes in brains of *Prnp*$^{o/o}$ mice (Brandner et al., 1996) and that PrP^{Sc} was not detectable in lethal scrapie-like disease in mice overexpressing mutant PrP transgenes (Telling et al., 1996), in some wild-type mice inoculated with BSE prions (Lasmezas et al., 1997; Manson et al., 1999) or in mice with a P101L mutation in their PrP genes (Manson et al., 1999). It is, however, not permissible to conclude from data of this kind that PrP^{Sc} as such is not infectious, because a 1000-fold reduction of the level of PrP^{Sc} found in wild-type, scrapie-ill mice would render it undetectable by commonly used methods, while a 1000-fold lower titer would still amount to $>10^5$ LD_{50} U/ml 10% brain homogenate.

"RML C4" prions resulting from RML-infected mice expressing PrPΔ32–93 gave similar incubation times as RML prions when they were inoculated into mice expressing the same transgene and efficiently transmitted disease to mice expressing full-length PrP; by these criteria, there is no evidence for a "prion transmission barrier" (Scott et al., 1989, 1993; Prusiner et al., 1990). However, RML C4 prions engendered moderate astrocytosis in the brainstem of *C4/C4* mice, which was not found after challenge with RML prions, possibly indicating some difference in their properties. Because the Chandler isolate-derived RML is a strain mixture (Kimberlin et al., 1987b), it is not clear whether such a change would reflect selection of a minority population or modification or de novo generation of a strain.

In contrast to our PrPΔ32–93 lines described here, a line of transgenic mice expressing PrP with deletions of amino acids 23–88 at a similar level as ours were resistant to RML prions when on a PrP knockout background (Supattapone et al., 1999). Surprisingly, when on a *Prnp*$^{o/+}$ background, the same truncated PrP appeared to be permissive for mouse RML prions, as evidenced by the accumulation of truncated PrP^{Sc}. The authors suggest that full-length PrP acts in trans, possibly by a direct interaction with the truncated PrP. However, it is also possible that the high levels of prions and PrP^{Sc} accumulating in mice with a *Prnp*$^{o/+}$ background (Büeler et al., 1994) entrain conversion of the truncated PrP, a process that may be occurring at only an undetectable level on a *Prnp*$^{o/o}$ background, where the inoculum disappears from the brain almost completely within days (Büeler et al., 1993).

There are three differences between the transgenic lines we describe and these so-called Tg(MHM2,Δ23–88) *Prnp*$^{o/o}$ mice that could account for their different susceptibility to scrapie. First, our PrPΔ32–93 construct preserves the nine amino-terminal residues (23–31) of the mature protein that are lacking in the product encoded by the MHM2 PrP(Δ23–88) ORF. This domain comprises a highly conserved motif (KKRPKP) and has been implicated in a dominant-negative effect on PrP^{Sc} formation in cell culture (Zulianello et al., 2000). Second, the chimeric MHM2 PrP(Δ23–88) contains two amino acid substitutions in the "hamster domain" H, in positions 108 and 111. Third, five residues (89–93) that are absent in our PrPΔ32–93 are present in MHM2 PrP(Δ23–88). We can currently not determine which of these differences or combination of differences is critical.

While PrP devoid of the octarepeats sustains scrapie-like disease in response to scrapie prions, the course and presentation of disease are profoundly modified. In particular, it is striking that animals die of a neurological disease without the histopathological changes in the brain typical for mouse scrapie. It is possible that the mice suffer from a disease that is confined to the spinal cord or to some restricted "clinical target areas" in which, it was suggested, scrapie agent must replicate for lethal disease to develop (Kimberlin et al., 1987a). It is, however, also possible that the critical events in pathogenesis occur at the submicroscopic or molecular level and that vacuolation, astrocytosis, and extensive neuronal cell death are late features not essential for clinical disease. These conclusions are complementary to the ones drawn in the case of scrapie-infected *Prnp*$^{o/+}$ mice, where strong astrocytosis, PrP^{Sc} deposition and high prion titers were found to be compatible with absence of clinical disease for over 30 weeks (Büeler et al., 1994). The molecular basis of scrapie pathogenesis remains as elusive as ever.

Experimental Procedures

Generation, Identification, and Maintenance of Transgenic Mice

The PrPΔ32–93 reading frame was introduced into the "half-genomic" PrP vector (Fischer et al., 1996) as described (Shmerling et al., 1998). Fertilized oocytes from *Prnp*$^{o/o}$ mice were injected to generate the *C4* line (Brinster et al., 1985; Wilmut et al., 1991). Microinjection of the construct into oocytes resulting from a cross between *Prnp*$^{o/o}$ and wild-type C57BL/6 mice yielded the *C15* founder. Transgene-positive founders were mated to *Prnp*$^{o/o}$ mice and two transgenic lines designated *C4/*− and *C15/*− were established from F1 progeny on a *Prnp*$^{o/o}$ mixed background C57BL/6 × 129/Sv (Büeler et al., 1992). Further breeding yielded the homozygous lines *C4/C4* and *C15/C15*. Transgene copy numbers were estimated relative to *Prnp*$^{o/o}$ alleles by quantitative PCR as detailed earlier (Shmerling et al., 1998).

Western Blot Analysis

Brain homogenates (10%, w/v) were prepared in PBS, 0.5% NP40, 0.5% sodium deoxycholate by passing brains through 18 gauge and 22 gauge needles. After centrifugation at 1500 × g for 10 min, supernatants were adjusted to 8 mg/ml of total protein. Where indicated, aliquots were digested with 20 μg/ml proteinase K for 30 min at 37°C, adjusted to 2 mM PMSF, and boiled in SDS-PAGE loading buffer with β-mercaptoethanol. The samples (40 μg or 80 μg of total protein in Figure 1A and Figure 1B, respectively) were electrophoresed through 16% SDS-polyacrylamide gels (NOVEX, San Diego) and transferred to PVDF membranes. PrP was detected with monoclonal antibody 6H4 (1:10.000, Prionics AG, Switzerland [Korth et al., 1997]) and Alzheimer precursor protein (APP) with the monoclonal antibody 22C11 (1:5000, Boehringer Mannheim, Germany). Blots were incubated with horseradish peroxidase–conjugated anti-mouse IgG1 antibodies (1:5000, ZYMED, San Francisco), developed using the enhanced chemiluminescence kit SuperSignal West Pico (Pierce, Rockford), and exposed to BIOMAX MR-1 film (Kodak, Rochester). An appropriate film exposure was scanned with a laser densitometer (Molecular Dynamics) and quantified using ImageQuant software.

Phosphotungstate Precipitation

Brain homogenates (10% w/v, in PBS) from *Prnp*$^{o/o}$, uninfected *C4/C4*, RML-infected, terminally ill wild-type, as well as *C4/C4* mice were prepared as above and centrifuged at 1500 × g for 10 min. An aliquot of each wild-type or *C4/C4* supernatant (1.8 mg total protein) was mixed with *Prnp*$^{o/o}$ supernatant to give a final volume of 0.5 ml and NaPTA precipitation of the abnormal form of PrP was

performed by the method of Safar et al. (1998), as modified by J. Wadsworth (personal communication). For Figure 1C, final pellets were resuspended in 32 μl 8 M urea sample buffer (SDS-PAGE loading buffer, 4% β-mercaptoethanol, 20 mM methylamine) and boiled for 15 min. Aliquots (22 μl) or fractions thereof as indicated were analyzed by Western blotting as described above. For Figure 1D, pellets were resuspended in 15.2 μl PBS, 0.1% Sarkosyl and digested with 50 μg/ml proteinase K for 1 hr at 37°C. PMSF was added to 2 mM and loading buffer with β-mercaptoethanol was added to a final volume of 32 μl. After boiling, aliquots of 22 μl or fractions thereof as indicated were analyzed by Western blotting.

Histology and Immunohistochemistry

For histology, mouse brains were fixed for at least 24 hr in 4% paraformaldehyde in PBS, immersed for 1 hr in 98% formic acid, postfixed for 72 hr in 4% paraformaldehyde/PBS, and embedded in paraffin. Sections (5–8 μm) were stained with hematoxylin–eosin. Immunohistochemistry was performed with commercial antibodies (DAKO, Glostrup, DK) to GFAP (glial fibrillary acidic protein; 1:300) or synaptophysin (1:40). PrP was detected on microwave-treated sections using monoclonal antibody 8H4 (1:200) (Zanusso et al., 1998) and visualized using the peroxidase-anti-peroxidase (DAKO, Glostrup, DK) or the Tyramide Signal Amplification kit (NEN, Life Science, Boston) according to the manufacturers' instructions. Sections were counterstained with hematoxylin.

Scrapie Infection and Determination of Infectivity

RML, a mouse-adapted scrapie isolate (Chandler, 1961), was passaged in Swiss CD-1 mice (Charles River Laboratories). Inocula stocks were 10% (w/v) homogenates of RML-infected, terminally ill CD1 mouse brains in 0.32 M sucrose. Mice were challenged i.c. with 30 μl of a 10-fold dilution of the stock in PBS containing 5% bovine serum albumin (BSA). Infectivity of tissues was determined by inoculation of 30 μl samples of the indicated dilutions into right parietal lobe of homozygous *tga20/tga20* mice (Fischer et al., 1996). Prion titers were estimated from time elapsed to terminal disease (Prusiner et al., 1982; Brandner et al., 1996) or by endpoint titration (Reed and Muench, 1938).

Grafting Procedure and Infection of Brain Grafts

Embryos of *tga20/tga20*, *C4/C4*, or *Prnp*$^{o/o}$ mice were harvested at day 12.5 after conception and transferred into modified Hank's medium containing 10% fetal calf serum (FCS) and 2% glucose at 4°C. The neuroectodermal anlage was grafted into the caudoputamen of adult *Prnp*$^{o/o}$ mice where it fully differentiated into normal neuronal and glial components (Isenmann et al., 1996). Engrafted mice were inoculated i.c. in the hemisphere contralateral to the graft with 30 μl of 1% RML-infected brain homogenate 4 weeks after transplantation of *C4/C4* or *Prnp*$^{o/o}$ neuroectoderm. Mice bearing *tga20/tga20* grafts were inoculated 12 weeks after transplantation. Animals were monitored at weekly intervals for the first 50 days p.i. and at 2 day intervals thereafter. Brain grafts were recovered and assayed for infectivity by inoculation of a 1% homogenate into indicator mice as described (Brandner et al., 1996).

Histoblotting

Histoblots were prepared as described (Taraboulos et al., 1992; Brandner et al., 1996). Coronal frozen sections (12 μm) were mounted on nitrocellulose membranes. Total PrP and, after digestion with 100 μg/ml proteinase K for 4 hr at 37°C, protease-resistant PrP^{Sc} were detected using monoclonal PrP antibody 6H4 (Korth et al., 1997) (Prionics AG, Switzerland; diluted 1:1000 in 1% nonfat milk) and alkaline phosphatase immunoconjugates. Visualization was performed using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride (Boehringer Mannheim, Germany). In Figure 5, total PrP and protease-resistant PrP^{Sc} were detected with polyclonal rabbit anti-PrP R340 (Brandner et al., 1996) as described above.

Morphometric Analysis

Spinal cord segments were fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. Serial 10 μm sections were stained with hematoxylin–eosin or with a monoclonal antibody against microtubule-associated protein 2 (MAP-2, 1:1000, Boehringer Mannheim, Germany). Motor neurons were identified by their location in the ventral horn of the spinal cord (Rexed laminae VIII and IX) and by MAP-2 staining. Cells were counted in the ventral horn of the cervical and lumbar spinal cord at 400× magnification in 15 focal planes at 100 μm intervals, as described (Clarke and Oppenheim, 1995).

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